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HAPTENIC PROPERTIES OF PARALYTIC SHELLFISH POISON *761100

Third Quarterly Report of Progress

on

Research Project Number 4B04-14-004
Order Number FDO-5013

January 1 - March 31, 1961

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Cincinnati, Ohio
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Unclassified Reports:

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HAPTENIC PROPERTIES OF PARALYTIC SHELLFISH POISON

I. Introduction

In keeping with the basic aims of this project, major emphasis continues to be given to the synthesis of conjugates of paralytic shellfish poison and protein, and to the evaluation of the haptenic properties of the PSP portion of the resulting antigens. Collateral studies are also in progress to investigate some chemical reactions of PSP in support of the syntheses and in an effort to develop alternate methods of assay.

In previous reports (1, 2) the results of two attempts at production and immunological evaluation of conjugates of PSP and protein have been presented. In the first experiment, failure to elicit an antibody response was attributed to the high toxicity of the preparation, while in the second, failure to obtain a haptenic response from the PSP portion of the molecule appeared to be related to an insufficient amount of diazotized PSP coupled to the protein. In this study, however, some evidence was developed to suggest the possibility that PSP did have haptenic properties.

This report summarizes the results of a third attempt at the synthesis, and immunological evaluation of PSP conjugates of ovalbumin and bovine gamma II globulin, in which the problems previously encountered were at least partially resolved. Attention has been given, also, to the nature of the changes brought about in PSP through the diazotization

reaction and to a new color reaction which may have some promise for the basis of an assay for PSP.

The studies anticipated on the physical properties of PSP-protein conjugates and alternate methods of coupling were not extensively pursued during this reporting period because of delays in obtaining parts for the electrophoresis apparatus and because of a limited supply of poison during this time, respectively.

II. Experimental

Preparation of non-toxic conjugates of PSP and protein - Modification III.

For each of the antigen preparations, 54.5 mg PSP were reacted with 2 g $NaNO_2$ and 2.5 ml 2 N HCl in a total volume of 20.5 ml for 35 minutes at 25°C to form the "diazo-poison" intermediate. At the end of this period sufficient solid Na_2CO_3 was added to make the solution approximately pH 9. Following this, 3 ml of a 10% protein solution (either ovalbumin or bovine II gamma globulin) contained in 0.1 M Borax buffer at pH 9.35 was added. Helium gas was bubbled through the mixture for 5 minutes to remove dissolved oxygen and to guard against alkaline oxidation of the diazotized PSP. The vessels were immediately sealed and placed in a water bath at 37°C. Protein control solutions were made in identically the same manner, except that no diazotized PSP was added. At the beginning of the incubation both the PSP ovalbumin and the PSP-gamma II globulin were orange in color while the respective protein controls were pale yellow. All solutions were incubated at 37°C for 40 hours. At the end of this time the PSP-proteins were a very dark amber and the controls were still pale yellow. Each solution was then dialyzed at 25°C for 10 hours against 3

liter volumes of physiological saline during which time the saline was renewed four times at 2.5 hour intervals. Although some of the yellow color was dialyzed from the solutions, the PSP-proteins remained a deep amber while the protein controls remained pale yellow. The final products were diluted to a total volume of 60 ml, divided into approximately 5 ml aliquots, frozen and stored for use in the immunological studies. Bio-assays with mice indicated that none of the solutions were toxic when injected at a level of 1/2 ml per mouse.

Details concerning the production of diazotized poison and other conditions for synthesis or for conjugation may be found in the second quarterly report.

Immunological studies related to determining the antigenicity of PSP-ovalbumin and PSP-bovine gamma II globulin.

The PSP-ovalbumin and PSP-bovine gamma II globulin described in the previous section were employed to immunize two groups of three rabbits each by intravenous injection (lateral ear vein). Previous work (2) revealed that no appreciable difference in antibody titers was obtained among rabbits injected intravenously or intramuscularly with adjuvent; therefore, only the former route of immunization was employed in the present studies.

Two additional groups of three rabbits each were immunized (I.V. injections) with ovalbumin and bovine gamma II globulin, in order to obtain a source of control sera. Because the coupling procedure described in the previous section was conducted at an alkaline pH, the ovalbumin and globulin, used to obtain control sera, were treated in a manner identical to the proteins coupled to PSP.

Five days prior to receiving the first injection of antigen, all 12 rabbits were bled by cardiac puncture to obtain samples of normal serum. The immunization schedule employed to obtain immune sera is shown in Table 1.

The sera collected from the above rabbits were tested for their precipitin content by mixing 0.4 ml of a 1:5 dilution of each of the sera with doubling dilutions of antigens from 1:2 through 1:8192. The mixtures were incubated in precipitin tubes in a 37°C water bath for one hour, followed by overnight incubation at 5°C. After incubation in the cold, the tubes were centrifuged at 2,000 r.p.m. for 5 minutes and observed for the amount of precipitate formed. Controls consisted of normal and immune serum-saline systems and antigen-saline systems. Primary titrations revealed that a non-specific precipitate developed in those tubes containing conjugated antigens in the higher concentrations. Therefore, in subsequent tests dilutions of antigens of 1:2 through 1:8192 were mixed with normal serum in order to determine the highest dilution at which the non-specific precipitate occurred in the control systems.

The results of initial titrations of anti-PSP-ovalbumin and anti-ovalbumin sera against PSP-ovalbumin, ovalbumin, and PSP-globulin antigens yielded unsatisfactory results. Before definite conclusions can be drawn concerning the type and degree of antibody response developed in these sera, several titrations will be necessary.

The results obtained with antisera produced to globulin and PSP-globulin, as well as the complete titration protocol, are shown in Tables 2 and 3. The data presented in Table 2 represent the results of an initial titration and Table 3 of a repeat titration with the same sera. Sera

numbered 36, 37, and 41 were produced against PSP-globulin in three rabbits, and their titers to the homologous antigen were in excess of 1:20,580. These sera also reacted with globulin and some precipitate was observed with all three sera at as high a dilution as 1:10,240. The significant titration depicted in Table 2 is that of anti-PSP-globulin sera 36, 37, and 41 with PSP-ovalbumin antigen. These titrations reveal that a cross reaction occurred, and the end points of the demonstrable reactions were 1:320 for serum 36, 1:640 for serum 41, and 1:1,280 for serum 37. These appear to be specific reactions in that pre-immunization sera (normal sera) of rabbits 36, 37, and 41 did not react with PSP-ovalbumin, PSP-globulin, or globulin alone. The results of normal serum titrations are also shown in Table 2. In that the only factor in common between anti-PSP-globulin and PSP-ovalbumin is PSP, a haptenic response appears to have been obtained, and all three sera contain antibodies capable of reacting with the PSP portion of the conjugated ovalbumin antigen.

Table 2 also shows the results of titrations of one of the anti-globulin sera (48) against globulin, PSP-globulin, and PSP-ovalbumin antigens. The antibody titer to globulin and PSP-globulin is lower than expected. The alkalinization of the globulin probably resulted in some denaturation which affected the antigenicity of the protein. The interesting point is that the coupled globulin (also alkalinized) is a better antigen than the altered globulin alone.

The titrations shown in Table 2 were repeated and the results of the second titration are shown in Table 3. The titer of anti-PSP-globulin sera for PSP-globulin and globulin alone were slightly lowered. However, the cross reaction to PSP-ovalbumin observed in the first titration did not

occur. A third titration of anti-PSP-globulin to PSP-ovalbumin again resulted in negative results. In view of the relatively high titers observed in the first titration, the negative results of the latter titrations are puzzling. The amount of precipitate and the nature of the floc developed initially in all three serum-antigen systems were typical of a precipitin reaction and were readily distinguishable from the non-specific flocculation observed in the antigen-saline and antigen-normal serum control tubes. In addition, the reaction occurred at too high a titer to be dismissed as non-specific. The results are uninterpretable at the moment; however, one explanation may lie in the manner the sera were treated. To date, the practice has been to freeze the sera in bulk rather than small aliquots for storage. This required alternate freezing and thawing which may have affected adversely the antibodies directed toward PSP. Comparison of the results shown in Tables 2 and 3 reveals a slight decrease in titer for each of the sera in the second set of titrations (Table 3). The reaction of anti-globulin (48) to globulin and PSP-globulin, though poor in the first titration, practically disappeared in the second titration. Though alternate freezing and thawing for one or two times usually is not considered deleterious to antisera, the possibility is worth further investigation. To determine whether alternate freezing and thawing is responsible for the loss of reaction, rabbits 36, 37, 41, and 48 have been given booster injections of 1.0 ml. The sera from these rabbits will be collected and separated into three aliquots. One aliquot is to be titrated immediately after collection (fresh serum), a second aliquot is to be preserved with merthiolate and stored in the cold, and the third aliquot is to be frozen. Comparative

titrations among the variously treated aliquots may shed light on the loss of specificity to PSP by the sera.

Studies on diazotized paralytic shellfish poison.

Since all of the conjugated antigens prepared to date have employed the use of PSP modified with nitrous acid prior to coupling, knowledge of the changes in the molecule brought about by this treatment are of interest.

It is well known that some of the reactions of nitrous acid with aliphatic nitrogen containing compounds, notably N-nitrosation, are reversible and that certain others, as in the formation of aliphatic diazo compounds, are not. Hence an investigation was undertaken to determine whether PSP enters into reversible equilibrium with nitrous acid. This was accomplished by the initial preparation of the essentially non-toxic yellow diazo-PSP in the presence of excess nitrous acid followed by removal of the excess nitrous acid by addition of sulfamic acid. Mouse bioassays were performed daily on the resulting solution and appropriate controls. It was found that toxicity of the solution increased with respect to time, thus indicating the regeneration of the poison. At the present time reversal has been accomplished to about 50% of theoretical. This evidence indicates that PSP enters into a reversible chemical equilibrium with nitrous acid and that the PSP molecule is not drastically altered by this treatment.

Color reactions of PSP.

It was previously reported (1) that PSP, after treatment with nitrous acid and removal of excess nitrous acid by addition of sulfamic acid, couples with β-naphthol to produce a red to pink color. It was also reported that PSP couples with aromatic diazonium salts (2) with production of a yellow color. It has recently been found that PSP also

reacts with sodium 1,2-naphthoquinone-4-sulfonate at pH 9.3 with production of a weak violet color and strong U.V. absorption at 242 mμ. The molar absorption coefficient at 242 mμ is 15,400 1/mole.cm., and PSP has been determined in concentrations as low as 7 μg in 0.5 ml of solution. This coupling occurs directly without any prior treatment of the poison. A similar reaction occurs with many compounds containing activated methylene and primary and secondary amine groups, which may render the reaction unsuitable for assay purposes. Teritary amine groups do not interfere however.

III. Projected Research for Fourth Quarter, FY 1961

Projected research for the fourth quarter, FY 1961, will be directed toward the following objectives:

1. Continued investigation of the immunological properties of PSP-protein (Modification III) in an effort to unequivocally determine if the PSP portions of the conjugates have haptenic properties.
2. Development of more rigorous evidence concerning the nature of the PSP-proteins through the use of physical techniques such as electrophoresis and ultracentrifugation.
3. Continued studies on the development of chemical micro-assay procedures for PSP.
4. Investigation of alternate methods for coupling proteins to PSP.

IV. Summary

Through additional modification of the procedures for coupling diazo-PSP to proteins, a product has been synthesized of sufficiently

low toxicity to be acceptable for use as an antigen and containing at least 10 times the amount of PSP as previous preparations.

Immunological studies of PSP-bovine gamma II globulin, PSP-ovalbumin and their respective controls provided conflicting evidence regarding the haptenic properties of the PSP portion of the conjugates yet to be reconciled.

Evidence has been presented to indicate PSP enters into a reversible equilibrium reaction with nitrous acid and that the "diazotization" does not appear to drastically alter the PSP molecule.

In search of alternate methods for chemical assay procedures, a color reaction between PSP and sodium 1, 2, -napthoquinone-4-sulfonate at pH 9.3 has been investigated in terms of specificity and sensitivity.

V. References

1. Haptenic Properties of Paralytic Shellfish Poison, First Quarterly Report on Research Project Number 4B04-14-004, U.S. Army Chemical Corps Biological Laboratories. Robert A. Taft Sanitary Engineering Center, October 1960.
2. Ibid, Second Quarterly Report, January 1960.

Table 1

Immunization schedule for rabbits receiving intravenous injections of globulin, ovalbumin, PSP-globulin and PSP-ovalbumin

Day	Dose
1	0.1 ml.
3	0.2 ml.
5	0.3 ml.
8	0.5 ml.
10	1.0 ml.
17	Bled
19	1.0 ml.
26	Bled

Table 2

Reaction of Anti-PSP-globulin rabbit sera to various antigens (I.)

Serum diluted 1:5 - 0.4 ml. per tube	Antigen	No. of Serum	Dilutions of Antigens 0.4 ml. per tube												
			Undiluted	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048	1:4096	1:8192
Anti-PSP-globulin	PSP-globulin	36		2+	3+	4+	4+	4+	4+	4+	4+	4+	3+	2+	+	-
	PSP-globulin	37		4+	4+	4+	4+	4+	4+	4+	4+	4+	3+	2+	+	+
	PSP-globulin	41		4+	4+	4+	4+	4+	4+	4+	3+	3+	2+	+	±	-
Anti-PSP-globulin	Globulin	36		2+	2+	3+	3+	4+	4+	4+	4+	3+	2+	+	-	-
	Globulin	37		3+	3+	4+	4+	4+	4+	4+	4+	3+	2+	+	±	-
	Globulin	41		4+	4+	4+	4+	4+	4+	4+	4+	3+	2+	+	-	-
Anti-PSP-Globulin	PSP-ovalbumin	36		4+	4+	4+	4+	3+	2+	-	-	-	-	-	-	-
	PSP-ovalbumin	37		4+	4+	4+	4+	3+	2+	+	±	-	-	-	-	-
	PSP-ovalbumin	41		4+	4+	4+	4+	2+	2+	+	-	-	-	-	-	-
Anti-PSP-globulin	Saline	36	-													
	Saline	37	-													
	Saline	41	-													
	PSP-globulin	Saline		+	±	+										
	Globulin	Saline		-	-	-										
	PSP-ovalbumin	Saline		±	±	-										
Normal serum	PSP-globulin	36		±	-	-										
	Globulin	36		±	-	-										
	PSP-ovalbumin	36		±	-	-										
Normal serum	PSP-globulin	37		±	-	-										
	Globulin	37		±	-	-										
	PSP-ovalbumin	37														
Normal serum	PSP-globulin	41		±	±	±										
	Globulin	41		-	-	-										
	PSP-ovalbumin	41		±	-	-										
Normal serum	PSP-globulin	48		±	-	±										
	Globulin	48		-	-	-										
	PSP-ovalbumin	48		-	-	-										
Normal serum	Saline	36	-													
	Saline	37	-													
	Saline	41	-													
	Saline	48	-													
Anti-globulin	Globulin	48		±	+	2+	3+	2+	2+	2+	+	-	-	-	-	-
	PSP-globulin	48		±	+	2+	3+	2+	+	+	+	±	-	-	-	-
	PSP-ovalbumin	48		±	+	+	-	-	-	-	-	-	-	-	-	-
	Saline	48	-													

Table 3

Reaction of Anti-PSP-globulin rabbit sera to various antigens (II.)

Serum diluted 1:5 - 0.4 ml. per tube	Antigen	No.of Serum	Undiluted	Dilutions of Antigens												
				1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048	1:4096	1:8192
Anti-PSP-globulin	PSP-globulin	36		2+	3+	3+	4+	4+	4+	4+	4+	3+	2+	±	-	±
	PSP-globulin	37		2+	3+	4+	4+	4+	4+	4+	4+	4+	3+	+	±	±
	PSP-globulin	41		4+	4+	4+	4+	4+	4+	3+	3+	2+	+	+	-	-
Anti-PSP-globulin	Globulin	36		2+	3+	3+	4+	4+	4+	4+	4+	3+	2+	+	-	-
	Globulin	37		3+	3+	3+	3+	4+	4+	4+	4+	3+	2+	+	-	-
	Globulin	41		4+	4+	4+	4+	4+	4+	3+	3+	2+	+	-	-	-
Anti-PSP-globulin	PSP-ovalbumin	36		-	-	-	-	-	-	-	-	-	-	-	-	-
	PSP-ovalbumin	37		-	-	-	-	-	-	-	-	-	-	-	-	-
	PSP-ovalbumin	41		-	-	-	-	-	-	-	-	-	-	-	-	-
Anti-PSP-globulin	Saline	36	-													
	Saline	37	+													
	Saline	41	-													
	PSP-globulin	Saline		±												
	Globulin	Saline		+												
	PSP-ovalbumin	Saline		±												
Normal serum	PSP-globulin	36		-	-	-	-	-	-	-	-	-	-	-	-	-
	Globulin	36		±	±	-	-	-	-	-	-	-	-	-	-	-
	PSP-ovalbumin	36		±	-	-	-	-	-	-	-	-	-	-	-	-
Normal serum	PSP-globulin	37		±	-	-	-	-	-	-	-	-	-	-	-	-
	Globulin	37		±	-	-	-	-	-	-	-	-	-	-	-	-
	PSP-ovalbumin	37		-	-	-	-	-	-	-	-	-	-	-	-	-
Normal serum	PSP-globulin	41		±	-	-	-	-	-	-	-	-	-	-	-	-
	Globulin	41		-	-	-	-	-	-	-	-	-	-	-	-	-
	PSP-ovalbumin	41		-	-	-	-	-	-	-	-	-	-	-	-	-
Normal serum	PSP-globulin	48		-	-	-	-	-	-	-	-	-	-	-	-	-
	Globulin	48		-	-	-	-	-	-	-	-	-	-	-	-	-
	PSP-ovalbumin	48		-	-	-	-	-	-	-	-	-	-	-	-	-
Normal serum	Saline	36	-													
	Saline	37	-													
	Saline	41	-													
	Saline	48	-													
Anti-globulin	Globulin	48		±	+	+	+	+	+	+	±	-	-	-	-	-
	PSP-globulin	48		±	+	+	+	+	+	+	±	-	-	-	-	-
	PSP-ovalbumin	48		-	-	-	-	-	-	-	-	-	-	-	-	-
	Saline	48	-													